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Leukocyte–endothelial adhesion is impaired in the cremaster muscle microcirculation of the copper-deficient rat

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Abstract

Dietary copper deficiency impairs the function of both the vascular endothelium and circulating leukocytes. In the current study, leukocyte–endothelium adhesion was observed in the in vivo cremaster muscle microcirculation of copper-adequate and copper-deficient rats. Male, weanling Sprague–Dawley rats were fed purified diets that were either adequate (5.6 μg/g) or deficient (0.3 μg/g) in copper. Adhesion was stimulated with the inflammatory mediators tumor necrosis factor-α and bradykinin, and the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine. Intravascular adhesion of leukocytes to the vascular endothelium was significantly attenuated in the copper-deficient group in response to all three agonists. These results occurred without any difference in intravascular wall shear rate between the dietary groups. Based on previous work, we propose that the attenuated response is caused by either decreased expression of adhesion molecules on leukocytes and endothelial cells or by inhibition of the endothelial cell calcium signaling associated with copper deficiency.

Keywords: Copper; Leukocyte adhesion; Microcirculation; Endothelium

1. Introduction

Dietary copper deficiency alters the responses of several effector cells involved in the circulatory component of inflammation. These include decreased endothelium-derived nitric oxide (NO)-mediated vasodilation, increased mast cell-mediated macromolecular leakage and attenuated platelet–endothelium adhesion [1].

In addition, leukocytes (specifically neutrophils) are particularly sensitive to restriction of dietary copper. In a study of neutrophils from copper-deficient mice, decreased expression of surface markers GR-1 and CD11b suggested that the circulating neutrophils were immature [2,3]. Neutrophils from rats fed copper-marginal and copper-deficient diets had impaired function including the respiratory burst and aerobic killing of bacteria [4,5]. Interestingly, this functional impairment occurred without marked alterations in the traditional indicators of copper status [5].

The microvascular response to inflammatory stimuli includes the infiltration of leukocytes from the blood to sites of inflammation. Initially, this adhesion process involves a series of events that occur along the vessel wall. Transient interactions between leukocytes and activated endothelium ('rolling') are typically required for the subsequent firm attachment of leukocytes to ('sticking') and transit across the vascular wall [6]. The initial capture and rolling of leukocytes by the endothelium is primarily mediated by a class of lectin-containing surface adhesion molecules collectively termed 'selectins'. L-Selectin is constitutively expressed on the surface of leukocytes [7]. P-Selectin is constitutively expressed in membranes of endothelial Weibel–Palade bodies and is rapidly mobilized to the luminal endothelial surface upon activation [8]. E-Selectin is synthesized de novo and expressed on the luminal endothelial surface following cytokine stimulation [9].

Firm adhesion or sticking of leukocytes to the endothelium is primarily mediated by the engagement of leukocyte integrins to members of the immunoglobulin superfamily on the endothelial cell surface. Intercellular adhesion molecule-1 (ICAM-1) is a counter receptor for

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the integrins and is constitutively expressed on resting endothelium [10]. However, the expression of ICAM-1 is elevated by inflammatory cytokines including those released from mast cells such as interleukin-1 and tumor necrosis factor (TNF)-α. These processes of rolling and adhesion of leukocytes precede their transendothelial migration from the vascular space into the interstitial space where the activated leukocytes release proteolytic enzymes, oxygen free radicals and other products for the destruction of invading organisms.

Previous studies have suggested that copper deficiency primarily affects the ability of the leukocytes to destroy bacteria [4,5]. If copper deficiency also causes the release of immature leukocytes [2,3], then we might expect that the ability of the leukocytes to adhere to the vascular wall, the first step for leukocytes to reach the bacteria, would also be impaired. In the current study, we examined the effect of copper deficiency on the adhesive interactions between leukocytes and the vascular endothelium in the in vivo microcirculation of the rat cremaster muscle. The endothelial adhesion response was stimulated with either the proinflammatory cytokine TNF-α or the endothelial receptor-dependent kinin bradykinin. Leukocytes were stimulated with the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). The role of dietary copper in leukocyte–endothelial adhesion was determined by comparing between groups fed copper-adequate and copper-deficient diets for 4 weeks.

2. Materials and methods

2.1. Animals and diet

This project was approved by the University of Louisville Animal Care and Use Committee. Male weanling Sprague–Dawley rats were purchased from Charles River Breeding Laboratories, Wilmington, MA. On arrival, rats were housed individually in stainless steel cages in a temperature- and humidity-controlled room with a 12-h light–dark cycle. The rats were given free access to distilled water and to one of two purified diets for 4 weeks. The basal diet [11] was a casein–sucrose–cornstarch-based diet (number TD 84469; Teklad Test Diets, Madison, WI) containing all known essential vitamins and minerals except for copper and iron. The copper-adequate (CuA) diet consisted of the basal diet (940 g/kg total diet) with safflower oil (50 g/kg) and a copper–iron mineral mix that provided 0.22 g ferric citrate (16% Fe) and 24 mg/kg diet $CuSO_4 \cdot H_2O$. The copper-deficient (CuD) diet was the same except for replacement of copper with cornstarch in the mineral mix. Diet analysis by atomic absorption spectrophotometry indicated that the CuA diet contained 5.56 mg copper/kg diet and the CuD diet contained 0.33 mg copper/kg diet. Parallel assays of National Institute of Standards and Technology (NIST) (Gaithersburg, MD) reference samples (citrus leaves, number 1572) yielded values within the specified range, which validated our copper assays.

2.2. Intravital microscopy

In preparation for in vivo experimentation, the rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). Thirty minutes prior to surgery, the rat was given an injection of sodium cromoglycate (5 mg/kg intravenously). Pretreatment with this mast cell stabilizer prevents leukocyte rolling caused by mast cell degranulation during surgical manipulation [12]. The trachea was cannulated to maintain airway patency and the carotid artery was cannulated to directly monitor blood pressure. The cremaster muscle was prepared for in vivo microcirculatory observation as previously described [13] and positioned over an optic port in a specially designed plexiglass bath. The bath was filled with 60 ml modified Kreb's solution [13] that was maintained at a temperature of 35 ± 0.5°C and a pH of 7.4 ± 0.5.

The rat and tissue bath were placed on the modified stage of a Nikon MM-11 microscope so that the microcirculation could be observed by transillumination of the cremaster muscle. Closed-circuit television microscopy was used to observe and quantitate the diameters of single, unbranched third-order venules that had basal diameters of 25–35 μm. The video system was calibrated with a stage micrometer and the vessel diameters were measured with a video caliper. Centerline red blood cell velocity was measured with an IPM model 102-B velocity tracker. Blood flow was determined from red blood cell (RBC) velocity and vessel diameter using the formula [14]:

$$\text{Flow (nl/s)} = \text{RBC velocity}/(1.6)(\pi r^2)(0.001)$$

Venular wall shear rate (SR) was calculated from the equation $SR = 8V/D$ where V is the centerline velocity and D is the internal vessel diameter.

The adhesive interactions between leukocytes and the vascular endothelium were determined by quantitating the number of transiently (rolling) and firmly (sticking) adherent leukocytes. A rolling leukocyte is defined as one that marginates along the vessel wall and is clearly dissociated from the bulk flow. A sticking leukocyte is one that stays stationary for 30 s. For each vessel, the number of rolling leukocytes passing an arbitrary reference point and the number of stationary leukocytes in a 100 μm segment of venule were counted for 1 min. Values are reported as the number of rolling leukocytes/60 s and the number of sticking leukocytes/mm^2 endothelial surface.

2.3. Experimental protocols

Following a 1 h equilibration period, mean arterial blood pressure, red blood cell velocity, vessel diameter and baseline values of rolling and sticking leukocytes were recorded. Topical administrations of 500 U TNF-α ($n=6$ CuA and $n=6$ CuD), 1.6×10^{-7} M bradykinin ($n=6$ CuA and $n=6$ CuD) or 10^{-7} M fMLP ($n=7$ CuA and $n=5$ CuD) were used to stimulate leukocyte–endothelial adhesion. TNF-α upregulates the expression of several endothelial adhesion molecules over a 4–6 h period [15] so measurements were made each hour for 6 h. Since both bradykinin and fMLP are short-acting agonists [16,17], measurements were made 30 min after treatment with these agonists.

2.4. Copper status indices

The median lobe of the liver was removed, weighed and frozen at −10°C for subsequent copper analysis. Tissues were lyophilized and digested in nitric acid and hydrogen peroxide [18]. Hepatic copper concentrations of individual rats were assessed using inductively coupled argon plasma emission spectrometry (model 1140; Jarrell-Ash, Waltham, MA). Parallel assays of reference samples (number 1477a, bovine liver) from the NIST yielded mineral contents within the specified range. Hematocrit was determined as an indirect index of copper deficiency [18].

2.5. Statistical analysis

Data are presented as mean ± S.E.M. Effect of diet on copper indices, rheological parameters, baseline leukocyte rolling and sticking values, and the leukocyte adhesion responses to bradykinin and fMLP were examined by one-way analysis of variance (ANOVA). Comparison of the change in leukocyte rolling and sticking values over time between dietary groups treated with TNF-α was by two-way repeated measures ANOVA. Differences were considered significant at $P<0.05$. If a significant difference was found by two-way ANOVA, Tukey's test was used to determine which means were different.

3. Results

Rats fed the copper-deficient diet for 4 weeks developed anemia and had significantly lower liver copper concentration compared with the copper-adequate fed control group (Table 1). These markers are indicative of copper deficiency. The copper-deficient diet did not affect the mean arterial blood pressure, venular diameters and rheological characteristics or the baseline adhesive interactions between leukocytes and the vascular endothelium (Table 1).

Table 1
Copper status and baseline values for rheological parameters and leukocyte–endothelial adhesive interactions[a]

	Copper-adequate ($n=12$)	Copper-deficient ($n=12$)
Copper indices		
Hepatic copper (μg/g dry weight)	12.67 ± 0.60	2.98 ± 0.49*
Hematocrit (%)	48.3 ± 1.1	33.2 ± 1.6*
Cardiovascular indices		
Mean arterial blood pressure (mmHg)	119 ± 8	120 ± 2
Venular diameter (μm)	33.2 ± 1.6	33.5 ± 2.1
Venular blood flow (μl/s)	0.96 ± 0.24	1.29 ± 0.20
Shear rate (s^{-1})	291 ± 65	402 ± 71
Leukocyte adhesion		
Rolling leukocytes (cells/min)	28 ± 4.2	47 ± 14.1
Sticking leukocytes (cells/mm^2)	35 ± 10.4	43 ± 8.0

[a] Values presented as mean ± S.E.M. * $P<0.05$.

Stimulation of the vascular endothelium with TNF-α caused a time-dependent increase in both rolling (Fig. 1) and sticking (Fig. 2) leukocytes in the in vivo microcirculation of copper-adequate rats as expected. However, the leukocyte–endothelial adhesion response was significantly less in the copper-deficient (Figs. 1 and 2) group as determined by two-way ANOVA ($P=0.018$ for rollers and $P=0.044$ for stickers). Endothelial stimulation with bradykinin also increased the rolling and sticking of leukocytes in post-capillary venules of cop-

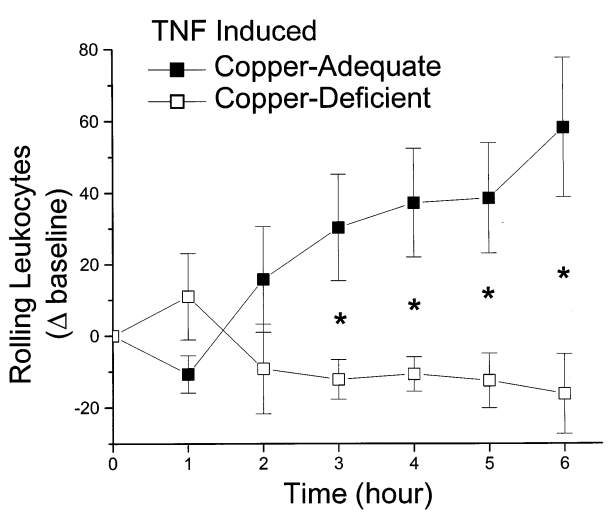

Fig. 1. The effect of TNF-α on rolling leukocytes in post-capillary venules of copper-adequate ($n=6$) and copper-deficient ($n=6$) rats. Data presented show the change in the number of rolling leukocytes from the baseline value (mean ± S.E.M.). * $P<0.05$ for comparison between dietary groups.

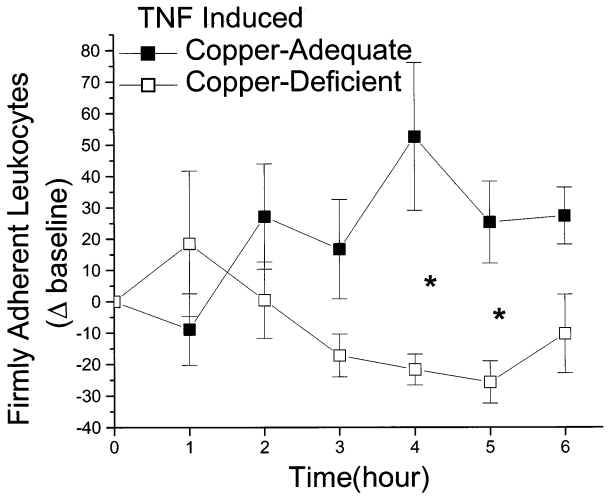

Fig. 2. The effect of TNF-α on firmly adherent leukocytes in post-capillary venules of copper-adequate ($n = 6$) and copper-deficient ($n = 6$) rats. Data presented show the change in the number of adherent leukocytes from the baseline value (mean ± S.E.M.). * $P < 0.05$ for comparison between dietary groups.

per-adequate rats (Fig. 3). In the copper-deficient group, the firm adhesion of leukocytes to the endothelium in response to bradykinin was significantly attenuated ($P = 0.038$) compared with the CuA group (Fig. 3) while rolling was unaffected.

Stimulation of the circulating leukocytes with the chemoattractant fMLP produced a decrease in rolling leukocytes and an increase in sticking leukocytes in the CuA group (Fig. 4). The number of rolling leukocytes decreased to a significantly greater extent in the CuD group ($P = 0.007$) than in the CuA controls (Fig. 4). While the number of sticking leukocytes increased in the CuA group, firmly adherent leukocytes decreased in the CuD group in response to fMLP (Fig. 4). The

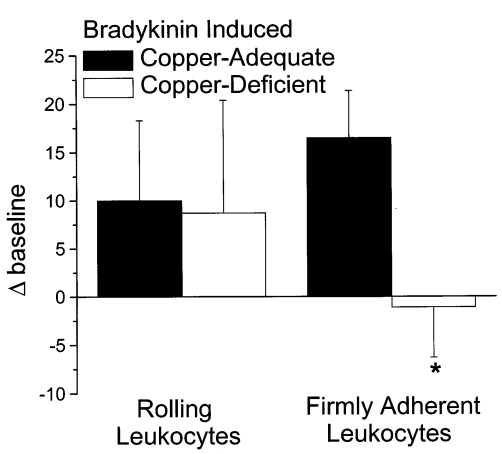

Fig. 3. The effect of bradykinin on rolling and firmly adherent leukocytes in post-capillary venules of copper-adequate ($n = 6$) and copper-deficient ($n = 6$) rats. Data presented show the change in the number of rolling and adherent leukocytes from the baseline values (mean ± S.E.M.). * $P < 0.05$ for comparison between dietary groups.

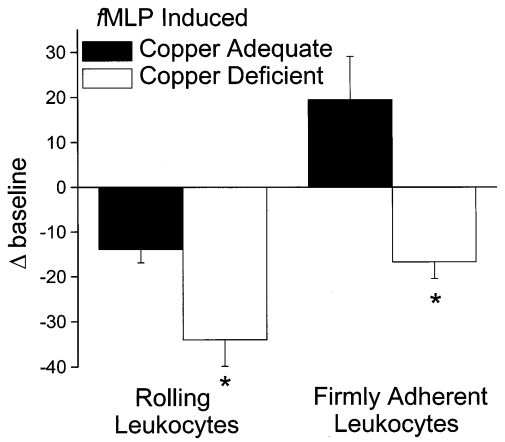

Fig. 4. The effect of fMLP on rolling and firmly adherent leukocytes in post-capillary venules of copper-adequate ($n = 7$) and copper-deficient ($n = 5$) rats. Data presented show the change in the number of rolling and adherent leukocytes from the baseline values (mean ± S.E.M.). * $P < 0.05$ for comparison between dietary groups.

difference between dietary groups for sticking leukocytes was significant ($P = 0.013$).

4. Discussion

Copper deficiency has a marked effect on the function of circulating leukocytes. Even under conditions of marginal copper deficiency when traditional indicators of copper status are not altered, the respiratory burst and bacteriacidal mechanisms of neutrophils are diminished [4,5]. Similar results have been reported in differentiated human U937 promonocytic cells after copper chelation [19]. There is also evidence that the peripheral neutrophils are immature during copper deficiency. Decreased expression of cell surface markers GR-1 and CD-11b on neutrophils from copper-deficient mice [2,3] suggests that maturation is arrested. In addition to indicating that these copper-deficient cells are immature, depressed expression of CD-11b (the α-subunit of the MAC-1 neutrophil integrin that binds to ICAM-1) suggested that leukocyte–endothelial adhesion could be attenuated.

In addition to impairment of leukocyte maturation and function, dietary copper deficiency also attenuates several microvascular control mechanisms related to endothelial cell function. We have shown that macromolecular leakage is increased because of mast cell degranulation, while endothelium-derived NO-mediated vasodilation and platelet-to-endothelial cell adhesion are diminished in the in vivo microcirculation of copper-deficient rats [1]. Recent data from copper-chelated human promonocytic cells demonstrate that the upregulation of CD54 (ICAM-1) was suppressed [20]. These results suggest that in addition to previously documented changes in the function of vascular endothelium

in copper-deficient rats [1], the expression of ICAM-1 on endothelial cells may also be depressed, leading to reduced leukocyte–endothelial cell adhesion.

The current study is the first to observe the interactions between leukocytes and endothelium in vivo during copper deficiency. The results demonstrate that under basal conditions, there is no dietary effect on either transiently or firmly adhered leukocytes (Table 1). However, when leukocytes or endothelial cells are stimulated, leukocyte adhesion to the vascular wall is attenuated in postcapillary venules of copper-deficient rats (Figs. 1–4). The endothelium was stimulated with TNF-α, which upregulates the surface expression of P-selectin [9] and ICAM-1 [21]. Bradykinin was also used to stimulate leukocyte adhesion to the post-capillary venule endothelium by a mechanism involving platelet-activating factor [16]. fMLP was used as a stimulant because it activates polymorphonuclear leukocytes without affecting the endothelium [22]. Therefore, the attenuated adhesion was not specific to a particular stimulation pathway.

There are several possible mechanisms for the diminished leukocyte adhesion seen in these in vivo experiments. The first is the reduced expression of adhesion proteins on the surface of both leukocytes and endothelial cells that have been shown to occur in copper-deficient neutrophils [2,3] and promonocytic cells [20]. Diminished expression of the selectins or ICAM-1 and/or the corresponding integrins would reduce the adhesion of leukocytes to the vascular wall prior to transmigration.

A second possible mechanism by which copper deficiency attenuates leukocyte adhesion involves the generation of the peroxynitrite anion ($ONOO^-$) during copper deficiency. Peroxynitrite is the product of the interaction of superoxide anion (O_2^-) with NO and has been shown to be elevated in the plasma of copper-deficient rats [23]. This probably occurs with a buildup of O_2^- because of the decreased activity of Cu,Zn-SOD during dietary copper restriction [18]. The increased activity of $ONOO^-$ is known to inhibit leukocyte–endothelial interactions by the inhibition of P-selectin [24]. Peroxynitrite may also inhibit leukocyte adhesion by reducing the endothelial cell calcium mobilization induced by cytotoxic leukocytes [25]. We have previously shown that endothelial cell calcium mobilization is reduced in the vascular endothelium of copper-deficient rats [23], which suggests that calcium-dependent signaling within endothelial cells is reduced during copper deficiency.

Leukocyte–endothelial adhesion may also be altered by changes in blood rheological properties including shear rate. Leukocyte adherence to the vascular endothelium depends on the effect of the adhesive forces generated by the leukocyte and the endothelium and the hydrodynamic dispersal forces such as blood flow velocity and shear rate that tend to move white cells away from the vascular wall [26]. Experimental increases in shear rate have also been shown to increase the expression of endothelial ICAM-1 [27], suggesting a pro-adhesive role for the mechanical factor. In a previous study, we found that shear rate did not account for the depressed platelet–endothelial adhesion seen in copper-deficient rats [28] and, in the current experiments, there was no difference in shear rate between the dietary groups (Table 1). Therefore, it is unlikely that changes in blood rheology had a role in the observed differences in leukocyte–endothelial adhesion between dietary groups.

While the current experiments demonstrate a diminished adhesion of circulating leukocytes to the vascular endothelium in copper-deficient rats independent of the stimulation pathway, other data suggest that copper deficiency promotes the adhesion of neutrophils to endothelium. Karimbakas et al. [2] found a significant increase in neutrophil specific myeloperoxidase activity in the lungs of copper-deficient mice after lipopolysaccharide (LPS) stimulation even though the circulating population of neutrophils was considered immature. Their results suggest that greater numbers of cells were sequestered by the lung in response to the inflammatory stimulus. The mechanism for increased accumulation of the immature neutrophils in the lung is not known but may be related to the neutrophilia observed in the copper-deficient mice [2].

Differences between the current data and those of Karimbakas et al. [2] may be related to several factors, including the vascular bed studied (cremaster striated muscle versus lung). Since the endothelium exhibits much regional heterogeneity, the endothelial cells at different sites may respond differently to the same stimuli [29]. The type, mode and duration of the inflammatory stimuli (local application of bradykinin and fMLP for 30 min or TNF-α for 1–6 h versus systemic application of LPS for 17 h) were also different between the studies. Although there does not appear to be a difference in the adhesive response between various agonists in the current study (Figs. 1–4), the length of the experiments is important since peak expression of specific adhesion molecules varies with time after inflammatory stimulation [15] Also, the effect of LPS given systemically may be confounded by a decrease in mean arterial blood pressure in the copper-deficient rats [30]. Further research needs to be carried out to determine tissue specific responses of copper-deficient leukocytes to inflammatory stimulation.

In summary, this is the first in vivo study of the interactions between circulating leukocytes and the endothelium of the vascular wall during dietary copper deficiency. The results show that, while there is no observable difference between dietary groups during baseline conditions, the leukocyte–endothelium adhe-

sive responses to inflammatory stimuli are blunted in the microcirculation of striated muscle during dietary copper restriction. This attenuation is probably caused by decreased expression of adhesion molecules on the surface of leukocytes and/or endothelial cells or by an inhibitory effect of $ONOO^-$ on P-selectin expression and endothelial cell calcium mobilization necessary for the adhesive process.

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